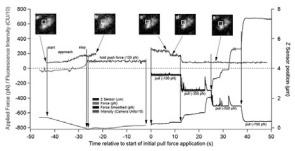
pulling force, as well as increases in the scanner z position sensor. Change in sensor position is consistent with changes in vesicle height as observed in the TIRF, based on the calibrated excitation decay constant (c-d). Rapid transients with small amplitude reveal partial tether extensions by 10-20nm, while large transients with slow decay reveal more gradual extensions, exceeding 1μm, that appear to be composed of multiple steps. These observations suggest that the dynamics of vesicle tether extensions are much more complex than previously thought. A large data set of recordings like that in the figure is being analyzed. Supported by NIH grant R21NS072577.



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The Role of Dense Core Nanoparticles in Regulation of Neuronal Communication

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The goal of this project is to introduce a novel bottom-up approach to probe the functionality of the dense core nanoparticles by creating artificial dense core nanoparticles from the native proteins.

Communication of neurons is happening through the exocytosis process, which is based on vesicular release of neurotransmitters like dopamine. A portion of these vesicles are large dense core vesicles and have the critical function of storing and excreting neurotransmitters or neuropeptides. The so-called dense core is composed of charged polypeptides from the chromogranin family of proteins. This protein matrix accumulates and stores neurotransmitters in high concentrations but the mechanisms of accumulation and release are unknown and highly debated.

So far in this work we have developed a model system for the first time as artificial dense core nanoparticles. The synthesized dense core protein nanoparticles are made of chromogranin A, which is the main protein, located in large dense core vesicles. This protein is proposed to involve in the storage of neurotransmitters, Ca^{2+}, and ATP within secretory vesicle. To explore the interactions between dense core proteins and neurotransmitters like dopamine, we are using isothermal titration calorimetry (ITC). By this study we could determine the binding affinity and stoichiometry of neurotransmitter storage under various physiological conditions, such as the pH gradient between the interior and exterior of the secretory vesicle, and the presence of other small physiological relevant molecules such as calcium and ATP.

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Fusion Properties of Gliotransmitter Vesicles in Astrocytes

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Astrocytes, the most abundant type of glial cells, play a number of important functions in information processing in the brain. They ensheath synapses and modulate synaptic transmission by many mechanisms which include the exocytotic release of gliotransmitters into the extracellular space. However, the anatomy and nature of exocytotic vesicle interaction with the plasma membrane is unclear. Using stimulated emission depletion (STED) and structured illumination (SIM) super-resolution microscopies, we studied the morphology of distinct gliotransmitter vesicles, whereas the interaction between a single vesicle with the plasma membrane was monitored by the high-resolution cell-attached patch-clamp measurements of membrane capacitance (Cm), a parameter linearly related to the surface area of the plasma membrane. Immunolabelling of vesicles containing D-serine, glutamate, atrial natriuretic peptide (ANP) and brain derived neurotrophic factor (BDNF) yielded their diameter to be < 100 nm, whereas ATP was found in larger vesicles (~200 nm diameter). Direct electrophysiologic measurements have shown that the predominant mode of vesicle interaction with the plasma membrane consisted of reversible capacitance steps, reflecting transient exocytosis, whereas irreversible capacitance steps, reflecting full-fusion exocytosis, were less abundant. The amplitude of discrete step increases in Cm reflects the size of interacting vesicles. By assuming the specific capacitance of 10 fF/μm2 and spherical morphology, the diameters of vesicles fusing with the plasma membrane ranged from 40 - 800 nm, consistent with the STED and SIM measurements. Reduction of extracellular Ca^{2+} attenuated the occurrence of unitary exocytotic events and treatment of astrocytes with Botulinum neurotoxins, which specifically inhibit the SNARE complex formation, attenuated the frequency of transient and full fusion events. These results show that in astrocytes the occurrence of unitary exocytotic events of small and large vesicles is Ca^{2+}- and SNARE- dependent.

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Phorbol Ester-Stimulated VWF Secretion from Human Umbilical Vein Endothelial Cells

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Von Willebrand factor (VWF) and the VWF-propeptide (proregion) are stored for secretion within specialized endothelial cell secretory organelles called Weibel-Palade bodies (WPBs). Because phorbol esters are potent VWF secretagogues and cause extensive WPB degranulation, it is generally assumed that PMA-evoked VWF secretion arises solely from WPB exocytosis (the VWF storage compartment). Here we present evidence that a significant fraction of the early phase of phorbol myristate acetate (PMA)-evoked VWF secretion from human endothelial cells arises from a cycloheximide (CHX)-sensitive (ie, nascent, non-storage) compartment. Stimulation for 15 minutes with either PMA (160nM) or histamine (100μM) caused similar amounts of VWF secretion. However, prior inhibition of protein synthesis (10μM CHX for 24 hours) reduced PMA-evoked secretion by ~70% while histamine-evoked secretion was unaffected. Optical analysis of live cells expressing a fluorescent protein targeted to WPBs showed that the first 15 minutes of PMA stimulation was associated with fusion of 8.4 $\pm$2.1% (mean$\pm$sem, n=11 cells) of fluorescent WPBs, compared to 41.4$\pm$4.0 % (mean$\pm$sem, n=12) for histamine. For both stimuli, the percentage of fluorescent WPB fusion events was unaffected by CHX treatment. Our data suggest that in addition to driving WPB exocytosis, PMA also causes acute release of non-stored VWF and proregion, most likely from the trans-Golgi network. The route and mechanisms regulating PMA-evoked release of non-stored VWF are under investigation.

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Ca^{2+} has a Permissive Effect on Glycolytic Oscillations in Pancreatic Beta Cells

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The secretion of insulin from pancreatic islet beta cells is pulsatile (~5 min period) and reflects upstream oscillations in metabolism and Ca^{2+}. Of the oscillating pathways in the beta cell, glycolytic oscillations are the least well studied. We recently developed a FRET biosensor for pyruvate kinase M2 activity (PKAR, Pyruvate Kinase Activity Reporter), which is activated by fructose 1,6-bisphosphate. We used PKAR FRET to measure oscillations in glycolysis stimulated by 10 mM glucose. After abolishing Ca^{2+} oscillations with diazoxide (Dz), the oscillations in PKAR were terminated. However, in some cases the oscillations could be restored by raising extracellular KCl, which elevated the intracellular Ca^{2+} levels but did not restore Ca^{2+} oscillations. These results indicate that glycolytic oscillations can persist in the absence of Ca^{2+} oscillations, and suggest the presence of a Ca^{2+} threshold that is permissive of glycolytic oscillations. To address this question, we varied the extracellular Ca^{2+} from 1.25 to 5 mM, which increased the amplitude of intracellular Ca^{2+} oscillations in a dose-dependent manner. Parallel measurements using PKAR indicated that the amplitude of glycolytic oscillations in pyruvate kinase M2 activity are augmented by intracellular Ca^{2+}, however PKAR oscillations were terminated if extracellular Ca^{2+} dropped too low. Taken together, these results indicate that glycolytic oscillations in beta cells are dependent upon on a threshold level of intracellular Ca^{2+} level, and represent a distinct oscillating compartment of the cell. Supported by R01DK46409 (L.S.).

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EHD3 Mediates Integral Membrane Protein Trafficking and Maintain Electrical Excitability and Adrenergic Responsiveness in the Heart

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Cardiac contraction relies upon the proper expression, trafficking, and retention of integral membrane proteins such as ion channels, transporters, and hormone receptors. These proteins govern cardiac contraction and short and long term adaptations to physiological and pathophysiological stimuli. The profile of expressed proteins is dynamic, and it is regulated to assure the proper

response to stress. Yet, despite its obvious importance, little is known regarding even the identity (or the function) of the intracellular molecular pathways underpinning the trafficking and targeting of integral membrane proteins in the context of the native heart. The goal of this work is identify new molecular players that regulate protein targeting and trafficking in the heart. Eps 15 homology domain-containing (EHD) gene products (EHD1-4) are intracellular proteins that are key regulators of endosomal trafficking, lipid homeostasis, membrane protein recycling and trafficking. Previously uncharacterized in the heart, we recently presented evidence which demonstrated that this protein family likely plays indispensable roles in protein trafficking in cardiac muscle. Notably, an essential role for one of these proteins, EHD3, in the membrane trafficking of the Na/Ca exchanger (NCX) in heart was uncovered. The goal of this research program is for the first time to directly test the role of these proteins in cardiac structural and electrical activity using cutting-edge *in vivo* models of EHD deficiency. We show that EHD3 deficiency in the heart leads to: 1) abnormal cardiac structure at baseline; 2) irregular action potential morphology, heart rhythm, and conduction; 3) depressed β-adrenergic responsiveness; 4) dysfunctional NCX and LTCC trafficking and function; and 5) dysregulated ankyrin-B expression and trafficking. These data strongly support a role for EHD3 in membrane protein trafficking and regulation within the context of the native heart.

Calcium Signaling II

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An Acidic Sequence in Neurogranin is Required to Modulate Ca^{2+} Binding to Calmodulin

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Background: Neurogranin (Ng) and PEP-19 are small proteins with no known intrinsic activity other than binding to calmodulin (CaM) via their IQ motifs, yet they have been implicated in numerous normal and pathological processes. We showed that PEP-19 is intrinsically disordered, and that an acidic sequence adjacent to its IQ motif is required for PEP-19 to modulate Ca^{2+} binding to CaM, and to sensitize HeLa cells to ATP-induced Ca^{2+} release. Goals: Ng has an acidic sequence, but with significantly different composition than PEP-19. Thus, the goals of the current study were to determine: 1) If the acidic sequence in Ng is required to modulate Ca^{2+} binding to CaM; and 2) Use NMR to compare the effects of Ng derivatives on the conformation of CaM. Results: Ng greatly increases the Ca^{2+} koff at the C-domain of CaM, but has little effect on the kon, thereby decreasing Ca^{2+} binding affinity. The peptide Ng(29-49), which includes only the consensus IQ motif does not increase the Ca^{2+} koff, but Ng(13-49), which includes the acidic region of Ng mimics the effect of intact Ng on Ca^{2+} binding to CaM. The 1H 15N HSQC spectra of Ng show it to be an intrinsically disordered protein. Effects of Ng protein and peptides on the NMR spectra of CaM are consistent with their relative effects on Ca^{2+} binding. Also, effects of Ng (29-49) on amide chemical shift perturbations and backbone dynamic properties of CaM are different from Ng or Ng(13-49), especially in the apo state. Conclusions: Modulating Ca^{2+} binding to CaM relies on the acidic region of both PEP-19 and Ng. Tuning Ca^{2+} mobilization pathways by PEP-19 and Ng expands the biological significance of these intrinsically disordered regulators of CaM signaling.

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Predicting Calmodulin Binding Sites via Canonical Motif Clustering

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The calcium-binding protein, calmodulin (CaM), directly binds to membrane transport proteins to regulate membrane excitability and calcium-dependent intracellular signal transduction cascades. Although 14 canonical CaM binding motifs have been defined, identifying CaM binding sites in membrane transport proteins is mostly a haphazard exploration, requiring mind numbing sequence gazing. Therefore, we developed a facile method for identifying all of the canonical CaM binding motifs within a given sequence. Analysis of the sequences from crystallized CaM-peptide structures revealed that CaM often binds to sequences that have multiple overlapping CaM binding motifs. Combining this algorithm with a simple charge discriminator results in reasonable predictive power using a test set of biochemically-characterized CaM binding motifs. Because we have found this analysis useful in both the design of experiments and analysis of experimental results, we have published the algorithm online and created a database that allows users to search for CaM binding motifs within a protein of interest, perform the meta-analysis, and then compare the results to target peptide-CaM structures deposited in the Protein Data Bank. The CaM binding motif algorithm, meta-analysis and website will be presented.

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Thermodynamic Analysis of Calmodulin Recognition of the Ion Channel Ryanodine Receptor

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In skeletal muscle, the calcium binding protein calmodulin (CaM) plays an essential role in excitation-contraction coupling by modulating the opening and closing of the calcium channel ryanodine receptor (RyR1). Biochemical studies have mapped the CaM binding site (CaMBD) to a short region on RyR1 comprising residues 3614-3643. By interacting with this region differently at high and low calcium, CaM acts as a 'switch', providing essential feedback in Ca^{2+} levels during muscle contraction. Our X-ray structure of Ca^{2+}-CaM bound to RyR1 CaMBD (2BCX) revealed a novel '1-17' motif of RyR1 hydrophobic amino-acids anchoring the CaM domains. In order to gain additional insights into the CaM-RyR1 interaction, we are pursuing thermodynamic studies of CaM-RyR1 CaMBD complexes at several Ca^{2+} concentrations.

The energy of interaction between CaM and RyR1 CaMBD was determined using Förster resonance energy transfer in an auto-fluorescent biosensor construct (YFP-CaMBD-CFP). Fluorescence titrations at increasing concentrations of CaM enabled us to determine association constants and free energies of binding at high and low calcium. Interestingly, the affinities of apo- and Ca^{2+}-CaM for RyR1 CaMBD differed by orders of magnitude (micromolar vs. subnanomolar). Mutational analysis was performed for RyR1 residues W3620 and F3636 which form close contacts with the CaM C- and N-domains in the 2BCX complex. Both in the presence and absence of calcium, mutation of W3620 to alanine resulted in significant effects on the binding affinity, while the F3636 mutation had weaker effects. Titrations with the individual CaM domains and CaM calcium-binding mutants show that the CaM C-domain is the main mediator of interaction. Future studies will further explore residue-specific differences in CaM-RyR1 recognition and the interplay between the processes of calcium- and target-binding to CaM.

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In Failing Cardiomyocytes, CaM-RyR2 Dissociation Leads to Defective Domain Interaction and Channel Destabilization

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Calmodulin (CaM) binding to RyR2 inhibits diastolic channel activity, indicating that CaM stabilizes RyR2 in the closed state. In conditions where CaM dissociates from RyR2, like heart failure (HF), RyR2 exhibits destabilized gating and increased activity. Those characteristics make CaM a critical regulator of RyR2 and potential therapeutic target for HF. Another leading mechanism for the RyR2 dysfunction in HF is defective domain interaction between N-terminal (N: 0-600) and central (C: 2000-2500) domains. However, the relationship between CaM-RyR2 binding and defective domain interaction in HF is unclear, especially in cardiomyocytes. Here, in cardiomyocytes from rat with HF induced by coronary ligation, we use FRET between fluorescent FKBP12.6 and CaM to specifically detect RyR2-bound CaM and measure the RyR2-CaM binding affinity. In steady-state binding affinity measurements, the Kd for CaM-RyR2 binding in HF myocytes is ~51 nM, which is ~3 fold increased vs. normal myocytes. By measuring the binding kinetics of fluorescent domain peptide DPc10 (F-DPc10), we can detect defective interaction between N-terminal and central domains in myocytes. In HF myocytes, the F-DPc10-RyR2 association rate was significantly accelerated vs. normal myocytes, indicating a destabilized domain interaction (unzipping). However, in HF myocytes, saturating RyR2 with high [CaM] dramatically reduced F-DPc10 binding to RyR2 (B_{max}) and greatly slowed the association rate (k_{on}). We conclude that in HF myocytes, reduced CaM-RyR2 binding affinity leads to defective domain interaction, shifting the channel to an ''unzipped'' state'. Promoting CaM-RyR2 reassociation can restore the defective domain interaction and stabilize the channel.